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Report

Regulation of Autophosphorylation Controls PLK4 Self-Destruction and Centriole Number

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Summary

Polo-like kinase 4 (PLK4) is a major player in centriole biogenesis: in its absence centrioles fail to form, while in excess leads to centriole amplification [1–5]. The SCF-Slimb/βTrCP-E3 ubiquitin ligase controls PLK4 levels through recognition of a conserved phosphodegron [6–13]. SCF-Slimb/βTrCP substrate binding and targeting for degradation is normally regulated by phosphorylation cascades, controlling complex processes, such as circadian clocks and morphogenesis [14]. Here, we show that PLK4 is a suicide kinase, autophosphorylating in residues that are critical for SCF-Slimb/βTrCP binding. We demonstrate a multisite *trans*-autophosphorylation mechanism, likely to ensure that both a threshold of PLK4 concentration is attained and a sequence of events is observed before PLK4 can autodestruct. First, we show that PLK4 *trans*-autophosphorylates other PLK4 molecules on both Ser293 and Thr297 within the degron and that these residues contribute differently for PLK4 degradation, the first being critical and the second maximizing auto-destruction. Second, PLK4 *trans*-autophosphorylates a phospho-cluster outside the degron, which regulates Thr297 phosphorylation, PLK4 degradation, and centriole number. Finally, we show the importance of PLK4-Slimb/βTrCP regulation as it operates in both soma and germline. As βTrCP, PLK4, and centriole number are deregulated in several cancers [14–17], our work provides novel links between centriole number control and tumorigenesis.

Results

The levels of PLK4 in *Drosophila* and human cells are regulated by the SCF-Slimb/βTrCP-E3 ubiquitin ligase complex [6–13]. PLK4 contains a conserved phosphodegron (DpSGXXpT), commonly found in Slimb/βTrCP substrates [6–13] (Figure 1A). Regulation of phosphorylation in the degron is crucial to control protein degradation and ultimately cellular and developmental processes [14].

Phosphorylation of two residues (Ser293 and Thr297) within the PLK4 degron is critical for degradation and depends on PLK4 activity. First, a PLK4 double-alanine mutant shows impaired affinity for Slimb/βTrCP, high protein stability, and centriole amplification [6–13]. Second, inactive PLK4 is more stable than the wild-type (WT) [6, 8, 9, 12]. Moreover, PLK4 autophosphorylates a region that includes the degron [6, 8, 9], but the identity of the phosphorylated residues is not determined. Therefore, it was suggested that autophosphorylation of PLK4 degron residues and/or residues surrounding the degron contribute to Slimb/βTrCP binding and consequent PLK4 degradation [6, 8, 9].

Although progress was made toward elucidating PLK4-induced autodestruction, we lack mechanistic understanding on its regulation, ensuring a window of time for PLK4 activity. It is not currently known which specific residues are involved and how their phosphorylation regulates PLK4 degradation and centriole number. Furthermore, it is debated whether this is a direct mechanism or mediated by another kinase (discussed in [8, 18, 19]). Understanding this complex mechanism is critical to elucidate centriole number control.

PLK4 Autophosphorylates Both the Ser293 and Thr297 within the Degron

We first validated that *Drosophila* PLK4 kinase activity is required for PLK4 degradation through Slimb. Pulse-chase experiments of ProtA-PLK4(WT) and PLK4(KD) (kinase dead) in the context of *PLK4* endogenous depletion and after inhibition of protein synthesis with cycloheximide showed that PLK4(KD) is more stable (Figure S1A–S1D available online). *Slimb* depletion led to an accumulation of ProtA-PLK4(WT) comparing with the GFP-negative control, but had minimal impact on the levels of ProtA-PLK4(KD) (Figure S1E), suggesting that Slimb-mediated PLK4 degradation depends on PLK4 activity. Moreover, unlike the WT counterpart [7–9, 11], ProtA-PLK4(KD) did not interact with Slimb or SkpA (Table S1). These data show that PLK4 activity is required for its degradation via Slimb.

To test whether PLK4 phosphorylates its own degron and which residues are involved, we developed phospho-specific antibodies against Ser293 and Thr297 (Figure S1F). Phosphorylation at both residues was absent upon coexpression of Lambda-Phosphatase (λppase [20]) (Figure S1F), and these antibodies did not recognize the corresponding single-alanine mutant (S293A and T297A) (Figure 1B and Figures 3F and 3G). To address whether PLK4 targets directly the degron residues, we expressed PLK4(WT) in the presence or absence of λppase, and KD in bacteria (Figure S1F). PLK4(WT) is highly autophosphorylated in bacteria, which is first evidenced by

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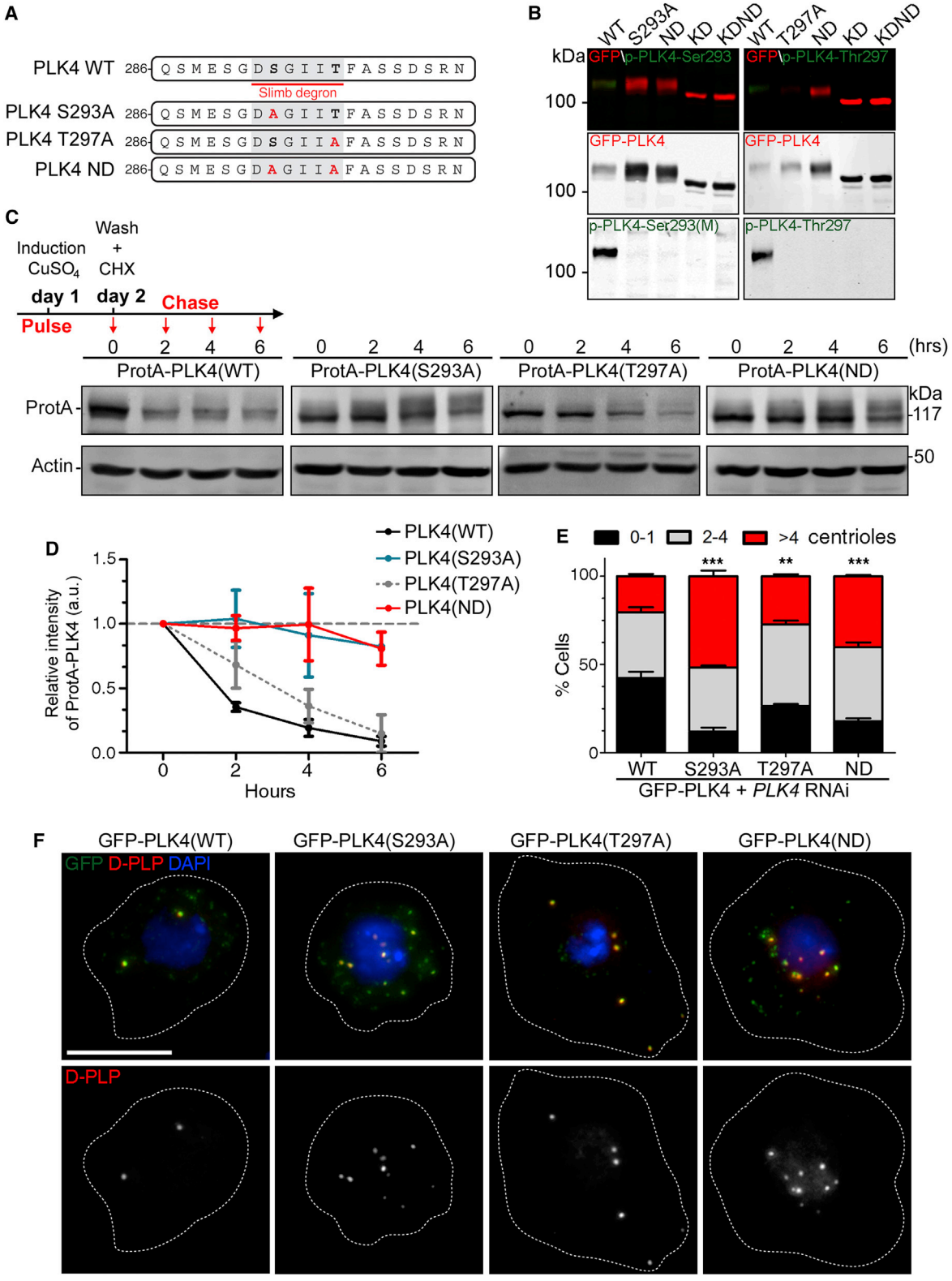

Figure 1. PLK4 Autophosphorylation of Ser293 Is Critical, while Autophosphorylation of Thr297 Maximizes the Efficiency of PLK4 Degradation and Centriole Number Control

(A) Consensus Slimb binding degron. Position of the first aminoacid residue is shown. Degron is highlighted in gray, and mutated Ser293 and/or Thr297 to alanine are shown in red. PLK4 WT, wild-type; PLK4 S293A, S293 mutated to alanine; PLK4 T297A, T297 mutated to alanine; PLK4 ND, nondegradable, both residues mutated to alanine.

(B) PLK4 autophosphorylates the degron in vivo. DMEL cells were transiently transfected with pMT-GFP-PLK4(WT), GFP-PLK4(S293A), GFP-PLK4(T297A), GFP-PLK4(ND), GFP-PLK4(KD), or GFP-PLK4(KDND) constructs after endogenous *PLK4* depletion and induced with 500 μM $CuSO_4$. Samples were

(legend continued on next page)

its slower mobility when compared to PLK4 coexpressed with λppase or compared with PLK4(KD) (Figure S1F, middle panels). Whereas PLK4(WT) is phosphorylated at both residues, phosphorylation is lacking in PLK4(KD), indicating that PLK4 autophosphorylates the degron (Figure S1F, lower panels). As kinases can extensively autophosphorylate within bacteria due to high concentrations and long incubations, we purified His-MBP-PLK4 that had previously been dephosphorylated in bacteria and followed its gradual autophosphorylation in vitro after adding ATP (Figure S1G). Autophosphorylation was observed early upon addition of ATP, showing that PLK4 autophosphorylates both residues within the Slimb degron in the absence of other eukaryotic kinases.

To investigate whether other kinases phosphorylate the degron in vivo, we evaluated PLK4 autophosphorylation in cultured cells expressing different GFP-PLK4 constructs after *PLK4* endogenous depletion (Figures 1B, S1C, and S1D). Whereas GFP-PLK4(WT) was phosphorylated at both degron residues, PLK4(KD) was not, showing that PLK4 activity is required for phosphorylation of both residues in vivo (Figure 1B). PLK4(ND) (nondegradable) mutant but not PLK4(KDND) displayed a broader band (Figure 1B), showing there is extensive PLK4-dependent phosphorylation of stable PLK4 variants in residues outside the degron.

Differential Requirement of Ser293 and Thr297 Phosphorylation for SCF-Slimb-Dependent Degradation of PLK4

Since PLK4 autophosphorylates both residues within the degron, we wondered what the impact of each of those events is on PLK4 stability. Many SCF/Slimb substrates have the typical DS/TG(X)2+nS/T degron. Both β-catenin degron phosphoserines (*DSGIHS*) form hydrogen bonds and electrostatic interactions with residues in the βTrCP-WD40 domains, suggesting a requirement for both residues concerning βTrCP docking [21]. However, this is not always the case, and the contribution of each phospho-residue for efficiency and timing of substrate degradation can vary [14, 22, 23]. To investigate how Ser293 and Thr297 contribute for PLK4 degradation, we analyzed the kinetics of degradation of ProtA-PLK4(S293A) and ProtA-PLK4(T297A). ProtA-PLK4(S293A) remained stable throughout the experiment [similarly to ProtA-PLK4(ND)], whereas ProtA-PLK4(T297A) showed slower degradation (Figures 1C and 1D), indicating different requirements for each residue. These results suggest that autophosphorylation of Ser293 is critical for PLK4 degradation, while autophosphorylation of Thr297 sets the speed at which PLK4 is degraded, determining its efficiency.

We investigated how the relative stability of each mutant impinges on centriole amplification. We expressed different GFP-tagged PLK4 variants, at very low levels from the leakiness of the pMT-promoter, in an RNAi background for endogenous *PLK4* (Figures 1E, 1F, S1C, and S1D) and used GFP-PLK4(ND) as positive control [7]. As expected, we observed a systematic increase in centriole amplification (more than four centrioles) with GFP-PLK4(S293A)-expressing cells relative to GFP-PLK4(WT) (Figures 1E and 1F). GFP-PLK4(T297A) elicited a modest, but significant, increase in centriole number compared with the WT, showing that maximum efficiency in PLK4 degradation is necessary to prevent deregulation of centrosome number (Figures 1E and 1F). The regulation of autophosphorylation of both residues is thus important for controlling PLK4 levels and ultimately centriole number.

PLK4 Phosphorylates Both Ser293 and Thr297 in *trans*

PLK4 is a suicide kinase, i.e., one whose activity determines its degradation through autophosphorylation. This is in contrast to many other processes regulated by Slimb/βTrCP, where several kinases phosphorylate the substrate [14, 22, 23]. How is PLK4 self-destruction regulated so that it is not immediately degraded? Perhaps phosphorylation of at least one degron residue is highly regulated by events dependent on PLK4, but independent of other eukaryotic proteins, as degron phosphorylation occurs in vitro (Figures S1F and S1G).

Interaction of PLK4 molecules in *trans* is important for PLK4 degradation, but it is not known which specific residues are *trans*-phosphorylated [6, 8, 9, 13], so we tested whether PLK4 can phosphorylate the degron in *trans* (Figure 2A). We coexpressed Myc-PLK4(WT) and GFP-PLK4(KD), the first in lower levels to preferentially examine the behavior of the KD (Figure 2B, right panel, left and middle lanes). When GFP-PLK4(KD) was coexpressed with GFP, it showed a defined band (Figure 2B, middle lanes), while coexpression with the WT led to a shift (Figure 2B, right lanes), accompanied by phosphorylation of Ser293 and Thr297 (Figure 2C, lower panels in each blot). These data suggest that PLK4 *trans*-autophosphorylates the degron in vivo.

It is possible that PLK4 phosphorylation in *trans* involves its oligomerization [8, 9, 13]. PLK4 has a triple polo box architecture [13] that facilitates oligomerization and targeting [8, 13, 24]. Homodimerization through PB1-PB2 promotes PLK4 binding to Slimb and degradation [8, 13].The fact that

analyzed by western blotting for p-PLK4 Ser293 (M) or p-PLK4 Thr297 and GFP (to detect total PLK4). Note that contrary to PLK4(WT), PLK4(KD) is not phosphorylated at both degron residues.

(C and D) Mutation of Ser293 to alanine blocks PLK4 degradation.

(C) pMT-ProtA-PLK4(WT), ProtA-PLK4(S293A), ProtA-PLK4(T297A), or ProtA-PLK4(ND) transfected DMEL cells were induced with $CuSO_4$ so that similar levels of protein where present at the beginning of the pulse chase. Cells were washed 18 hr later, incubated in medium supplemented with cycloheximide, and harvested at the indicated time points. Samples were analyzed by western blot for ProtA and actin (loading control).

(D) Quantification of ProtA-PLK4 relative intensity values shown in (C). These values were normalized to the actin loading control. Three independent experiments were used to generate the average for each time point. Error bars represent ± SEM. Note that while both ProtA-PLK4(S293A) and ProtA-PLK4(ND) remain stable, ProtA-PLK4(T297A) levels decrease with time but slower than WT.

(E and F) Phosphorylation of both Ser293 and Thr297 control centriole number.

(E) DMEL cells were transiently transfected with pMT-GFP-PLK4(WT), GFP-PLK4(S293A), GFP-PLK4(T297A), or GFP-PLK4(ND) constructs after endogenous *PLK4* RNAi. Due to the leaky nature of the pMT promoter, the experiments were conducted in a noninduced context. For scoring of centrioles, cells were fixed and stained against D-PLP and DNA (counterstained with DAPI). Data are the average of three independent experiments ± SEM (n = 100 cells in each experiment). Expression of PLK4(S293A), PLK4(T297A), and PLK4(ND) led to a statistically significant increase in centrosome number (more than four centrioles) when compared to PLK4(WT) (***$p < 0.001$, **$p < 0.01$; Pearson's $\chi 2$ test). *PLK4* RNAi results in an increase in cells with zero and one centrioles, a phenotype that is not fully rescued with expression of PLK4(WT) and is partially rescued with other more stable constructs.

(F) Representative images of cells analyzed in (E). GFP-PLK4 proteins are represented in green, D-PLP in red, and DNA in blue. Individual cells are outlined by dashed lines that represent the cell outline as judged by the D-PLP background signal. The scale bar represents 10 μm.

See also Figure S1 and Table S1.

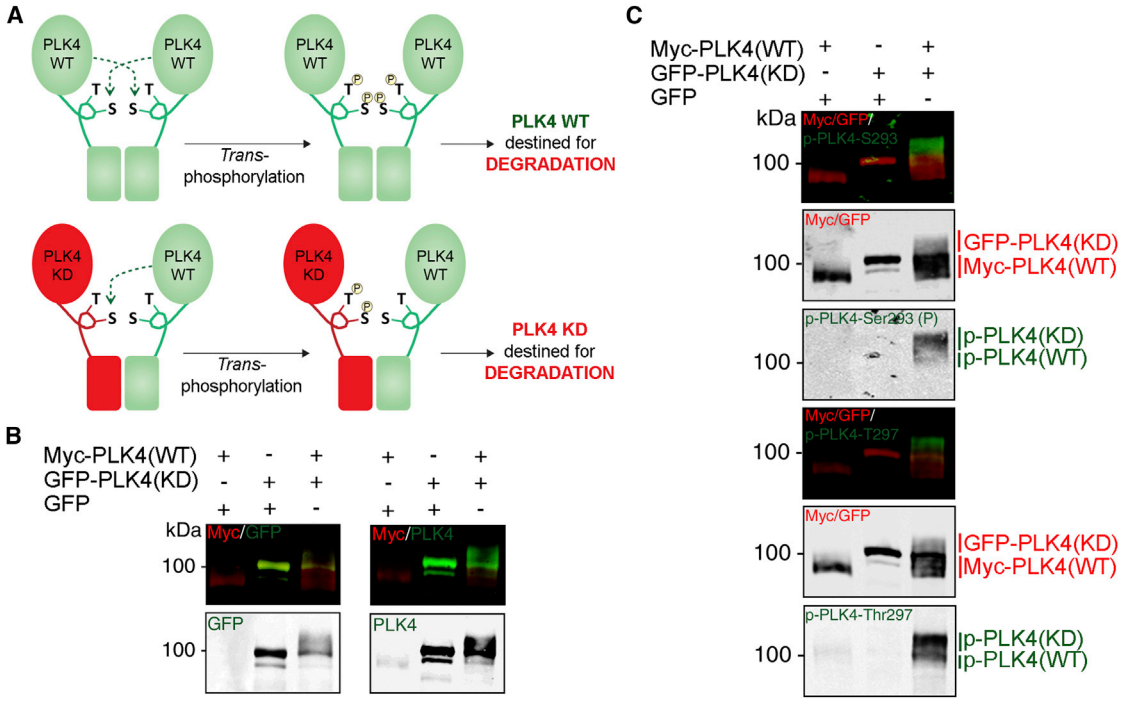

Figure 2. PLK4 *trans*-Autophosphorylates the Slimb Degron

(A) Schematic representation of PLK4 *trans*-autophosphorylation and expected regulation of protein levels. PLK4 has a triple polo box architecture that facilitates oligomerization and targeting [8, 13, 24]. *trans*-autophosphorylation might be dependent or not on dimerization through the polo boxes, in particular PB1-PB2 [8, 13, 24]. *trans*-autophosphorylation of two PLK4(WT) molecules leads to phosphorylation at both residues within the degron on both molecules. When PLK4(WT) dimerizes with a PLK4(KD) mutant, only the latter becomes phosphorylated within the degron.

(B and C) PLK4 *trans*-autophosphorylates the Slimb degron.

(B) Left: cell extracts were prepared and analyzed by western blotting for GFP [to detect pMT-GFP-PLK4(KD)] and Myc [to detect act5-Myc-PLK4(WT)]. Note that in the presence of Myc-PLK4(WT), GFP- PLK4(KD) becomes phosphorylated (lower mobility bands). Right: cell extracts were analyzed by western blotting for Myc and PLK4 antibodies to compare PLK4 levels under the act5 [PLK4(WT)] and pMT promoter [PLK4(KD)]. Note that PLK4(KD) is expressed 52-fold more than the WT.

(C) Combinations (as indicated) of act5-Myc-PLK4(WT), pMT-GFP-PLK4(KD), and pMT-GFP (empty) constructs were transfected in DMEL cells after endogenous depletion of *PLK4* (*PLK4*). The pMT constructs were induced with 500 μM $CuSO_4$ to ensure higher protein expression as compared to the act5 promoter. Coexpression of Myc-PLK4(WT) leads to phosphorylation of both Ser293 and Thr297 in a PLK4(KD) mutant. Note that since Myc-PLK4(WT) is expressed at much lower levels (B, right), it is impossible to detect the phosphorylation of this variant in (C).

See also Figures S1 and S2.

coexpression of a PLK4 mutant containing only PB1-PB2 leads to stabilization of PLK4(WT) and centriole amplification [8, 13] suggests that PLK4 targets the degron in *trans* within a dimer. Those experiments suggest that expression of PB1-PB2 alone sequesters full-length PLK4 through dimerization, preventing degron phosphorylation. To test whether dimerization through PB1-PB2 is necessary for degron phosphorylation, we coexpressed PB1-PB2 with PLK4(WT) (Figures S2A–S2D). Ser293, but not Thr297, phosphorylation was markedly reduced upon coexpression of PB1-PB2, suggesting that dimerization through the PB1-PB2 is only necessary for Ser293 *trans*-phosphorylation. We conclude that autophosphorylation within the degron is likely to be differentially regulated, with the critical Ser293 residue requiring dimerization through PB1-PB2. PLK4 thus needs to accumulate so that one molecule of PLK4 encounters another to dimerize and phosphorylate it at Ser293, the critical destruction residue.

A Phospho-Cluster around the Typical Slimb/βTrCP Degron Regulates Its Phosphorylation and Controls Degradation of PLK4

In other Slimb/βTrCP substrates, phospho-residues outside the conserved degron modulate the affinity of the substrate to the Slimb/βTrCP-WD40 positively charged domain and/or indirectly regulate degron phosphorylation [14, 22, 23]. *Drosophila* PLK4 has several putative phospho-residues around the *DSGIIT* degron (Figure 3A), several of which fit into the broad PLK4 phosphorylation consensus sites (reviewed in [18]). Six of those residues in *Drosophila* are conserved in murine PLK4, where they are autophosphorylated in vitro and are known to participate in PLK4 degradation, but it is not known which specific residues are phosphorylated [9] (Figure 3A). Mass spectrometry of in vitro reactivated PLK4 identified individual phosphorylation in at least five out of the six of those residues, including Ser293 and Thr297 (Figure 3A and Table S2). This phospho-cluster might thus play a critical role in regulating the kinetics of PLK4 binding to Slimb.

To address whether and how multisite phosphorylation impacts in PLK4 degradation and centriole number control, we mutated all conserved residues to alanine [PLK4(6A); Figure 3A] and observed that both Slimb binding and PLK4 degradation were impaired (Figures S3, 3B, and 3C). Moreover, PLK4(6A) leads to centriole amplification to a similar extent to PLK4(T297A) (Figures 3D and 3E) and localizes to centrioles (Figure 3D). These data indicate that phospho-cluster phosphorylation in *Drosophila* PLK4 is required for efficient binding

to Slimb and centriole number control. We further tested whether regulatory phosphorylations could enforce a sequence of events and found that phosphorylation of Ser293 does not depend on phosphorylation of Thr297, or vice versa (Figures 3F and 3G). However, while phosphorylation of Ser293 was not affected in PLK4(6A) (Figure 3F), phosphorylation of Thr297 was impaired (Figure 3G), suggesting that PLK4 phosphorylation around the degron primes PLK4 Thr297 autophosphorylation. The fact that Ser293 phosphorylation is well detected in the PLK4(6A) mutant, together with its proper centriole localization and ability to induce centriole formation (Figures 3D–3F), strongly suggests that those mutations do not substantially alter PLK4 structure and can thus be used to infer the behavior of the phospho-cluster. Increasing the phospho-occupancy around the degron thus regulates phosphorylation of Thr297, controlling PLK4 degradation efficiency.

Finally, we tested whether phospho-cluster phosphorylation also occurs in *trans*. We used priming of Thr297 phosphorylation as an indirect readout of phospho-cluster phosphorylation. We expressed Myc-PLK4(ND) with either GFP-PLK4(KD6A) or GFP-PLK4(KD) and asked whether phosphorylation of PLK4(KD6A) was impaired at Thr297 (Figures 3H and 3I). If phosphorylation of the cluster were to occur in *cis*, then neither GFP-PLK4(KD6A) or GFP-PLK4(KD) should be phosphorylated in the cluster when coexpressed with MycPLK4(ND) and both should show reduced levels of Thr297 phosphorylation. However, if phosphorylation of the phospho-cluster were to occur in *trans*, GFP-PLK4(KD6A) should show decreased Thr297 phosphorylation when compared to GFP-PLK4(KD). Indeed, we observed a marked decrease in phosphorylation at Thr297 in PLK4(KD6A) (Figures 3H and 3I). *Trans*-autophosphorylation of a phospho-cluster regulates Thr297 phosphorylation, suggesting both the need for a PLK4 concentration threshold and the presence of sequential events in timing PLK4 phosphorylation and degradation.

PLK4 Degradation in the Organism

Centriole number varies between tissues [25]. While cycling cells keep their number of centrioles constant between two and four, multiciliated cells assemble 200–300 centrioles/basal bodies [25]. In the female germline, centrioles disappear during oogenesis, after which centriole number is reconstituted during fertilization with the entry of the sperm centriole [25] (Figure 4A). In males, meiosis centriole duplication is uncoupled from the cell cycle (Figure 4D). PLK4 plays a universal role in centriole number control [1, 2, 4, 5, 27]. Its activity is essential for centriole formation in somatic cells [1, 2] and in the *Drosophila* germline [1, 4, 5, 27], and it correlates with the formation of multiple centrioles/basal bodies in multiciliated cells [28]. We investigated whether the SCF/Slimb controls PLK4 in different tissues. We created transgenic lines expressing either PLK4(WT) or PLK4(ND). The constructs were integrated by recombination within the same defined genomic loci to ensure the same mRNA expression levels between different lines, thus allowing for direct comparison between PLK4(WT) and ND protein levels [29].

PLK4 total levels in the female germline were 2-fold increased when its degradation was impaired (Figure 4B). We and others observed that centrioles disappear during oogenesis, even upon overexpression of high PLK4 levels [4, 5]; however, centrioles are formed de novo upon exit of meiosis [4, 5]. Overexpression of PLK4(ND) in eggs laid by virgin females led to a 2-fold increase in centrioles formed de novo (Figure 4C). The same results were observed with lines inserted in a different genomic locus (data not shown).

PLK4(ND) was expressed in the male germline using a promoter that becomes active before the meiotic divisions (Bam-Gal4). PLK4(ND) was very stable (Figure 4E) and was associated with centriole amplification (Figures 4F and 4G) and male sterility (Figure 4H). These data show that the PLK4 degron is functional in vivo in different tissues, suggesting that the SCF/Slimb complex generally controls centriole number.

Discussion

PLK4 is regulated by the SCF-Slimb/βTrCP-E3 ubiquitin ligase complex; without this control, several centrioles are generated per mother centriole [6–13]. Slimb/βTrCP-containing regulatory circuits normally operate using built-in time delays that couple protein degradation to different biological processes [14]. Simultaneous mutation to alanine of the two phosphorylatable residues within the PLK4-Slimb/βTrCP phosphodegron prevents PLK4 degradation [6–13]. Moreover, PLK4 degradation via Slimb/βTrCP was reported to be mediated by its own activity [6, 8, 9, 12]. Here, we show how PLK4 controls its degradation (Figure S4). *Drosophila* PLK4 autophosphorylates both Ser293 and Thr297 within the conserved degron (Figures 1 and S1). Each residue within the degron is regulated differently in a PLK4-dependent manner. While phosphorylation of both residues occurs in *trans*, only Ser293 depends on PLK4 dimerization (Figures 2 and S2). Moreover, while Ser293 is critical for PLK4 degradation, phosphorylation of Thr297 maximizes the efficiency of degradation (Figure 1). Finally, PLK4 *trans*-autophosphorylates a phospho-cluster that regulates Thr297 phosphorylation and binding to Slimb (Figures 3 and S3). In summary, two broad mechanisms are likely to ensure a window of PLK4 activity before it self-destructs: (1) *trans*-phosphorylating events ensure that a minimal amount of PLK4 accumulates before it targets itself for degradation and (2) a phospho-cluster ensures an order of phosphorylation events. Finally, we show that PLK4 and the SCF/Slimb complex control centriole number in different tissues, being essential in cultured somatic cells and in the germline in *Drosophila* (Figure 4).

PLK4 Self-Destructs

We show for the first time that PLK4 phosphorylates both Ser293 and Thr297 residues within the conserved SCF-Slimb/βTrCP destruction motif (Figure 1B and 3A and Table S2). Phosphorylation in PLK4(KD) was not observed in vivo (Figure 1B), showing that no other kinase can account for phosphorylation of those sites in a PLK4-independent manner. Degron phosphorylation with bacterially expressed-PLK4 (Figures S1F and S1G) shows that PLK4 can target these sites directly. Moreover, PLK4-reactivation experiments show that autophosphorylation in the degron can occur within a very short time in vitro, supporting its specificity (Figure S1G). It is still formally possible that in vivo, another kinase might target the degron in a PLK4-dependent manner. However, a kinome RNAi screen assaying PLK4 levels failed to produce a candidate [6], supporting our evidence that PLK4 regulates its own degradation in a direct manner.

Deciphering the PLK4 Degron, a Phospho-Cluster, and the Accuracy of PLK4 Degradation

SCF-Slimb/βTrCP-mediated degradation normally requires phosphorylation of both phospho-residues within a typical

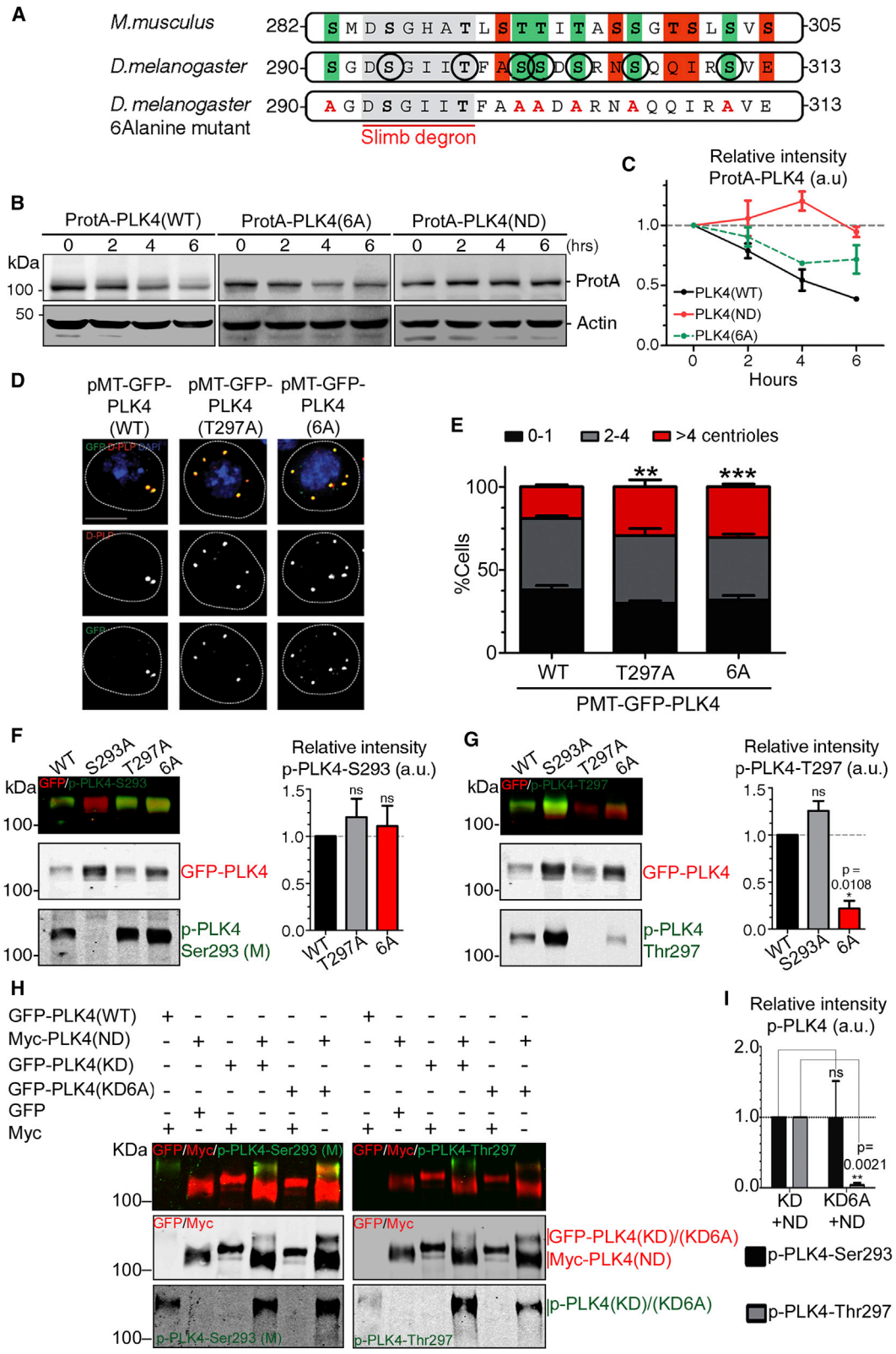

Figure 3. A Phospho-Cluster outside the PLK4 Degron Controls Phosphorylation within the Degron, PLK4 Degradation, and Centriole Numbers

(A) Alignment of *D. melanogaster* and *M. musculus* PLK4 24 amino acid domain [9] shows that six phospho-residues outside the degron are conserved. From these six residues, we identified five, plus the Ser293 and Thr297, as being autophosphorylated in vitro by *D. melanogaster* PLK4 (residues highlighted with a black circle; see Table S2 for more details on experiment and peptides identified by mass spectrometry). Color code: green highlight, conserved residues; red highlight, nonconserved residues (interestingly, some are substitutions of phosphorylatable residues by negatively charged residues). The sequence of the *Drosophila* PLK4 six alanine mutant is also represented. The positions of the first and last amino acid residues are shown.

(legend continued on next page)

degron [14], but each residue may have different roles in the regulation of degradation [14, 22, 23]. We show for the first time that Ser293 and Thr297 within the PLK4 degron have different contributions for PLK4 degradation: the Ser293A mutation rendered PLK4 as stable as the ND or KD form, whereas PLK4(T297A) showed slower degradation (Figures 1C, 1D, S1A and S1B). It is thus likely that Ser293 is the docking residue to bind Slimb, as in other substrates [30], being critical but not sufficient to ensure accurate PLK4 destruction. Thr297 phosphorylation might increase PLK4-Slimb affinity and thus maximizes the targeting and accelerates PLK4 destruction. In accordance with this, we found that Thr297A mutation alone impairs binding to Slimb (Figure S3). Individual phosphorylation of both residues is thus critical as expression of GFP-PLK4(T297A) or GFP-PLK4(S293A) led to significant centrosome amplification (Figures 1E and 1F).

We investigated a conserved phospho-cluster around the degron where most residues are phosphorylated in vitro by PLK4 (Figure 3A and Table S2). This phospho-cluster regulates phosphorylation of Thr297, as PLK4(6A) mutant showed impaired Thr297 phosphorylation in vivo (Figure 3G). This mutant shows less binding to Slimb, slower degradation and centriole amplification (Figures 3B–3E and S3). It is possible that those residues additionally regulate binding to Slimb/βTrCP in a Thr297-independent manner, perhaps by increasing the negative charges that interact with the positive charged amino acids in βTrCP/Slimb. A similar study [31] published in this issue also found that autophosphorylation of residues around the degron affects Plk4 stability and centriole number.

Mechanisms Coupling PLK4 Activity and Degradation

Here we uncovered two aspects of PLK4 degron autophosphorylation that may provide a window of time for PLK4 to promote centriole biogenesis before it autodestructs. First, since phosphorylation occurs in *trans*, a minimal concentration of PLK4 needs to be present before a PLK4 molecule encounters another and phosphorylates it. Additionally, the requirement for Thr297 regulatory phosphorylations, and the fact that they occur in *trans*, contributes to further delay full degron phosphorylation, ensuing timely PLK4 degradation and centriole number control.

How is PLK4 autoregulation linked to timing centriole biogenesis in the cell cycle? Our understanding of when in the cell cycle PLK4 regulates centriole duplication and how this is related to PLK4 levels, activity, and final targeting for destruction is still poor and needs further research. Both *PLK4* mRNA and protein levels increase from interphase to mitosis ([12, 32] and our unpublished data). Higher PLK4 levels in mitosis suggest that PLK4 attains higher activity at this stage and therefore is likely to be more phosphorylated at the degron. Since PP2A/Twins phosphatase counteracts Slimb-mediated degradation of PLK4 in mitosis [6], perhaps an additional layer of regulation is conferred by the concerted action of this and/or other phosphatases at both the degron phosphoresidues and phospho-cluster, preventing degradation.

Implications of PLK4 Degradation In Vivo

We show that the SCF/Slimb complex regulates PLK4 levels in vivo, being required for centriole number control in both the female and male *Drosophila* germline and impacting on their fertility (Figure 4). Neuroblasts from *Drosophila Slimb* mutants [33] and fibroblasts from *βTrCP*-mutant mice [34] show similar centrosome amplification, suggesting that regulation of PLK4 by this ubiquitin ligase is universally important in vivo. Centriole number is altered in the majority of cancers and its manipulation is widely discussed for diagnosis and therapeutics [14, 15, 17]. The knowledge that βTrCP controls centriole number in different tissues may offer new opportunities to interfere with centriole number in disease.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.09.037.

(B and C) Mutagenesis to alanine of the six conserved phospho-residues impairs PLK4 degradation.
(B) pMT-ProtA-PLK4(WT)-, GFP-PLK4(6A)-, or GFP-PLK4(ND)-transfected DMEL cells were induced with $CuSO_4$. Cells were washed 18 hr later, incubated in medium supplemented with cycloheximide, and harvested at the indicated time points. Samples were analyzed by western blotting for ProtA and actin loading control (the same experimental setup as indicated in Figure 1C).
(C) Quantification of ProtA-PLK4 relative intensity values shown in (B). These values were normalized to the actin loading control. The average of three independent experiments is shown. Error bars represent ± SEM.
(D and E) Phospho-cluster phosphorylation is required for centriole number control. Cells were transiently transfected with pMT-GFP-PLK4(WT), GFP-PLK4(T297A), or GFP-PLK4(6A) constructs in an endogenous *PLK4* RNAi background (*PLK4*). Due to the leaky nature of the pMT promoter, the experiments were conducted in a noninduced context. For scoring of centrioles, cells were fixed and stained against D-PLP and DNA (counterstained with DAPI).
(D) Representative images of cells where GFP-PLK4 proteins are represented in green, D-PLP in red, and DNA in blue. Individual cells are outlined by dashed lines. Note that the PLK4(6A) mutant localizes to centrioles. The scale bar represents 10 μm.
(E) Centriole counting of cells represented in (D). Note that expression of PLK4(T297A) and PLK4(6A) constructs led to a statistically significant increase in centriole number (more than four centrioles) when compared to PLK4(WT) (***$p < 0.001$, **$p < 0.01$; Pearson's $\chi 2$ test). RNAi for *PLK4* results in an increase in cells with zero and one centrioles, a phenotype that is not fully rescued with expression of PLK4(WT) and is partially rescued with other more stable constructs. Data are the average of three experiments ± SEM (n = 100 cells in each experiment).
(F and G) Phospho-cluster autophosphorylation regulates Thr297 phosphorylation. DMEL cells transiently transfected with pMT-GFP- PLK4(WT), GFP-PLK4(S293A), GFP-PLK4(T297A), and GFP-PLK4(6A) constructs after endogenous *PLK4* (*PLK4*) depletion were induced with 500 μM $CuSO_4$. Samples were analyzed by western blot for p-PLK4-Ser293 (F) or p-PLK4-Thr297 (G) and GFP antibodies (to detect total PLK4). The relative intensities of p-PLK4 Ser293 (F) or Thr297 (G) residues for each sample were obtained by normalization with the respective total PLK4 levels. Quantifications were performed using the *Odyssey* infrared image *system* (LI-COR). Data are the average of three experiments ±SEM (t test). Note that PLK4(6A) mutant shows impaired phosphorylation at Thr297 but not at Ser293 residue.
(H) Phosphorylation of the phospho-cluster occurs in *trans* in vivo. Combinations (as indicated) of pMT-GFP-PLK4(WT) (as a positive control for phosphorylation; low amounts are expressed), GFP-PLK4(KD), GFP-PLK4(KD6A), act5-Myc-PLK4(ND), pMT-GFP, and act5-Myc empty constructs were transfected in DMEL cells after endogenous depletion of *PLK4* (*PLK4*). The pMT constructs were induced with 500 μM $CuSO_4$. Note that individual antibody blots were used to annotate which protein is which in the figure.
(I) Quantification of relative phosphorylation at Thr297 in (H). The relative intensity of Thr297 for each sample was obtained by normalization with the respective total PLK4 levels. Quantifications were performed using the *Odyssey* infrared image *system* (LI-COR). Note that coexpression of Myc-PLK4(ND) with GFP-PLK4(KD6A) leads to a marked reduction in Thr297 phosphorylation when compared with GFP-PLK4(KD).
See also Figures S1 and S3 and Table S2.

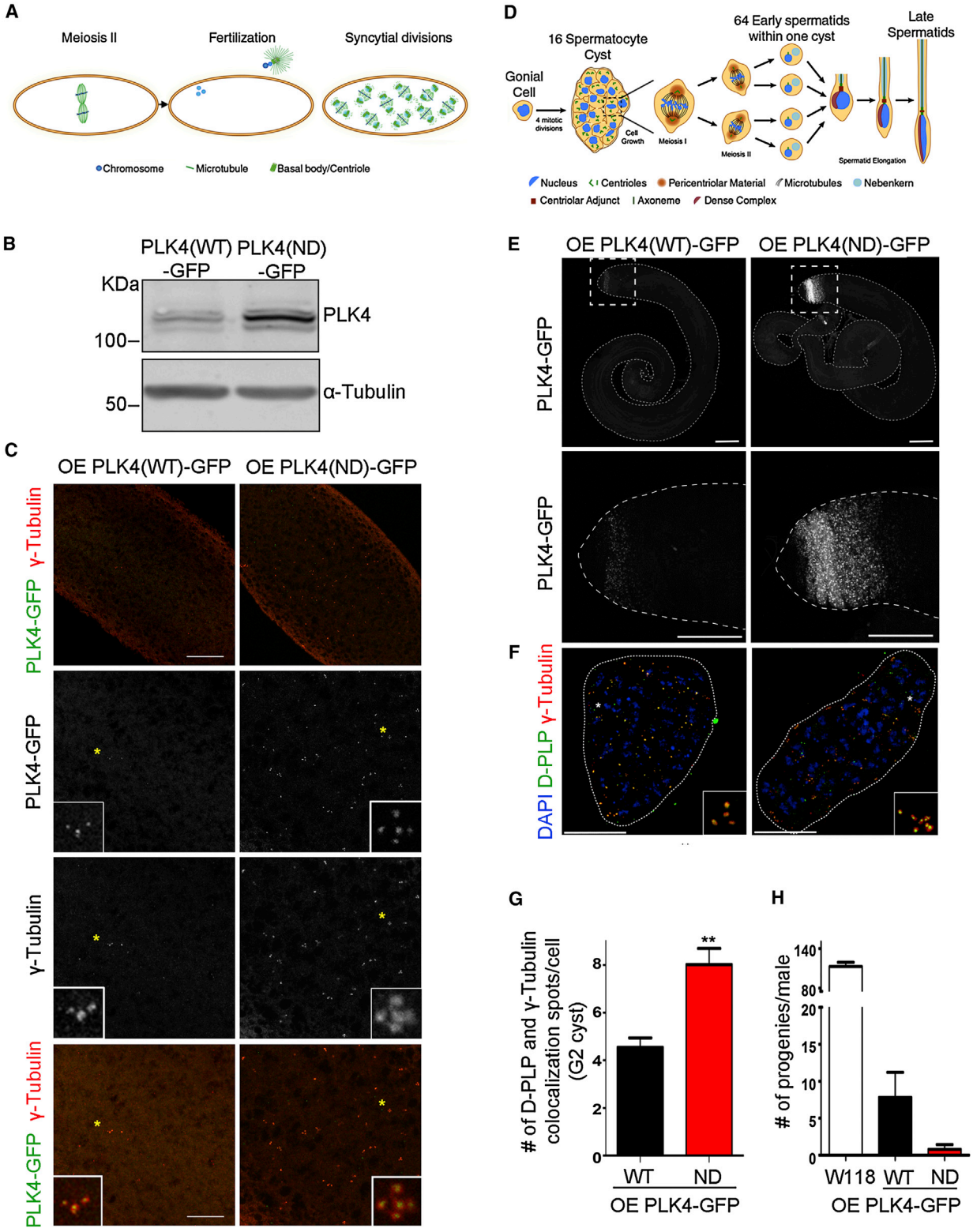

Figure 4. Slimb Degron Is Critical to Control PLK4 Stability and Centriole Number in the *Drosophila* Germline

(A) *Drosophila* early embryogenesis. Centrioles are lost during oogenesis. At fertilization, the sperm brings the first centriole of the egg and the two pronuclei meet. After the first mitotic division, syncytial divisions begin.

(B) PLK4(ND)-GFP is stabilized in *Drosophila* female germline when compared with PLK4(WT)-GFP expressed from the same promoter and genomic loci. *pUASp-PLK4(WT)-GFP* and *pUASp-PLK4(ND)-GFP* inserted by recombination in the same genomic loci were expressed under the control of the ovariole specific driver *V32Gal4*. Ovary extracts from 3-day-old females were analyzed by western blot with PLK4 antibody (α-tubulin was used as a loading control).

(C) Expression of PLK4(ND)-GFP leads to an increase in centriole de novo formation as compared to PLK4(WT)-GFP. Note that at 30 min after egg laying, eggs overexpressing PLK4(ND)-GFP show more centrioles as compared with the WT kinase (we observed a 2-fold increase after quantitation). A magnified

(legend continued on next page)

Author Contributions

I.C.-F. and I.B. did the majority of experiments and contributed equally to the work; A.P.-M and S.C.J. planned and did all the experiments in the organism and contributed equally to the work; M.L.-F., P.D., J.B.-P., S.G., T.A., D.B., and A.R.-M. generated tools and contributed with experiments; J.D. did the phosphomapping; N.D. helped with planning of experiments and optimization of protocols; I.C.-F., I.B., and M.B.-D. planned most of the experiments; and I.C.-F. and M.B.-D. wrote the manuscript. All authors discussed the work and manuscript.

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view of an area where centrioles are seen is shown. Red, γ-tubulin; green, PLK4-GFP. Scale bars represent (top) 25 μm and (bottom) 50 μm. The inset shows 5× magnification.

(D) *Drosophila* spermatogenesis. Cysts of 16 primary spermatocytes are formed upon the four mitotic divisions of the gonial cell. Each of these cells has four centrioles. In meiosis I each centrosome is built of two centrioles in a V shape, whereas in meiosis II each centrosome contains a single centriole. Meiotic divisions produce a cyst of 64 spermatids, each cell with one centriole (adapted from [26]).

(E) PLK4(ND)-GFP is highly stabilized in *Drosophila* male germline when compared with PLK4(WT)-GFP expressed from the same promoter and genomic loci. *pUASp-PLK4(WT)-GFP* and *pUASp-PLK4(ND)-GFP*, integrated at recombination site 28E7, were expressed under the male germline specific driver Bam-Gal4. Immunostaining of whole-mount testes shows that PLK4(ND)-GFP is stabilized in the male germline, being visible in more cells.

(F) Expression of PLK4(ND) in the male germline leads to centriole amplification. Representative images of G2 cysts expressing *pUASp-PLK4(WT)-GFP* and *pUASp-PLK4(ND)-GFP*, where centrioles were immunostained with *Drosophila* pericentrin like protein (D-PLP) and γ-tubulin. DAPI was used as DNA marker. Note the higher centriole amplification observed upon expression of PLK4(ND)-GFP as compared with WT kinase.

(G) Quantification of centriole number in G2 cysts. The average number of centrioles (labeled for D-PLP and γ-tubulin) per each cell in G2 cysts is represented. Data are the average of at least eight cysts (≥128 G2 cells) from each genotype ± SEM. Expression of PLK4(ND) led to a statistically significant increase in centrosome amplification when compared to PLK4(WT) (***$p < 0.001$; unpaired t test).

(H) Males expressing PLK4(ND)-GFP are sterile. The average number of progeny produced by male of depicted genotypes ±SEM ($p = 0.0021$, t test) is represented. Note that while males expressing PLK4(ND)-GFP are fully sterile, expression of PLK4(WT)-GFP from the same promoter only leads to partial sterility. W118, wild-type strain with no insertion.

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